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Functional Characterization of a Novel Disaccharide Membrane Transporter in the
Digestive Tract of the American Lobster, *Homarus americanus*

By

Olivia Rose Scheffler

A thesis submitted to the Department of Biology in partial fulfillment of the
requirements for the degree of Master of Science, Biology

UNIVERSITY OF NORTH FLORIDA

COLLEGE OF ARTS AND SCIENCES

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ABSTRACT

In animals, the accepted model of carbohydrate digestion and absorption involves reduction of disaccharides into the simple sugars glucose, fructose and galactose. Previous studies have shown the presence of disaccharides maltose and trehalose in the blood of several crab species, the crayfish and the American lobster. In 2011, a gene for a distinct disaccharide sucrose transporter (SCRT) was first found in *Drosophila melanogaster* and characterized using a yeast expression system. The purpose of the current study was to identify and characterize a putative disaccharide transporter analog in crustaceans using the American lobster, *Homarus americanus*. Brush border membrane vesicles purified from the hepatopancreas were utilized. After identification of a sucrose transporter in the brush border membrane of the hepatopancreas, transport kinetics experiments were used to characterize it using ^{14}C radio-labeled sucrose and a Millipore filter isolation technique. Lack of glycyl-sarcosine inhibition of sucrose uptake into vesicles indicated that the highly non-specific dipeptide transporter PEPT1 was not the functional transporter of sucrose. A more acidic pH of 4 was shown to drive sucrose transport in the absence of sodium. Sodium was then shown to also significantly stimulate sucrose uptake, which resulted in an overshoot at 1 minute over a hyperbolic potassium uptake curve, suggesting that both sodium and acidic pH were capable of driving disaccharide transport. Experiments that used a variety of monosaccharides and polysaccharides indicated that the disaccharides maltose and trehalose were the only sugars to significantly inhibit carrier-mediated sucrose transport (maltose $P = 0.017$, trehalose $P = 0.023$ using a one-way ANOVA) ($K_m = 0.1951 \pm 0.0630$ mM sucrose, $J_{\max} = 0.5884 \pm 0.0823$ nmol/mg protein \times 1 minute), suggesting specificity of the transporter. Sucrose in the presence of 20 mM maltose had a K_m of 0.5847 ± 0.1782 mM sucrose ($P = 0.030$) and a J_{\max} of

0.6536 \pm 0.1238 nmol/mg protein x 1 minute (P = 0.006). ANOVA P-values indicate the difference between the sucrose control curve and the maltose curve. The highly significant reduction between the K_m values of the control sucrose curve and the maltose curve suggests competitive inhibition between the two sugars. These two disaccharides could utilize the same transporter, and are appropriate for the physiology of the animal in this case, as lobsters commonly digest glycogen and chitin, polymers of maltose and trehalose, respectively. These findings suggest there is a brush-border proton-, or sodium-dependent, hepatopancreatic carrier process, shared by sucrose, maltose, and trehalose, that may function to absorb disaccharides that occur from digestion of naturally-occurring dietary constituents.

INTRODUCTION

One of the main functions of almost all body cells is the production of energy. This process requires simple carbohydrates D-glucose, D-fructose or D-galactose (Alvarado et al. 1984, Walmsley et al. 1998). In mammals, plants, fungi and a variety of invertebrates, the acquisition of nutrients and subsequent transmembrane transport of monosaccharide sugars into body cells to fuel this energy production has been well-characterized. In vertebrates, sugar polymers like sucrose are first digested by sucrase and other enzymes into monosaccharides before intestinal absorption. Absorption is conducted primarily by monosaccharide transporters, sodium-glucose linked transporters (SGLTs) and glucose transporters (GLUTs) (Meyer et al. 2011, Vitavska et al. 2013). These distinct transporters belong to two specific sugar transporter families, the human sodium-glucose cotransporter family (SLC5) and the major facilitator superfamily of membrane transporters (SLC2) respectively.

Transport of disaccharides is limited by size and permeability restrictions of these larger molecules. Disaccharides cannot undergo diffusion into cells, so protein carriers are utilized for transport of simple monomers instead. Sucrose is a disaccharide in which one glucose molecule and one fructose molecule are joined by an alpha 1-2 glycosidic bond. In mammals, it is well known that sucrose must first be broken down by hydrolysis into its components D-glucose and D-fructose before being transported across gut epithelial cell membranes (Walmsley et al. 1998, Obi et al. 2011). Work conducted with hamster intestines suggests that sucrose must be hydrolyzed along the intestinal wall before transport into the cell via SGLT1 and GLUT 5 (Alvarado et al. 1984). Previous work in an invertebrate also found the presence of a sodium dependent (SGLT1)-

like glucose co-transporter and localized a sodium-independent GLUT5-like fructose transporter in the brush border membrane of lobster intestines (Obi et al. 2011).

A few previous studies utilizing paper chromatography to analyze blood components of marine invertebrates found evidence of not only monosaccharides glucose, galactose and fructose present, but also disaccharides maltose and trehalose (Hu, 1958, Telford, 1968). These two disaccharides are appropriate for the physiology of the animals in this case, as marine invertebrate predators commonly digest glycogen and chitin, polymers of maltose and trehalose, respectively. Glycogen, a polysaccharide of glucose, serves as a major carbohydrate storage molecule and is found in high quantities in the liver and skeletal muscle of animals (Hers, 1976, Cohen, 1978). Chitin is a polymer of N-acetylglucosamine, another derivative of glucose, and is an important component of invertebrate shells and exoskeletons (Felse and Panda, 1999). Species tested in these studies included the purple shore crab, *Hemigrapsus nudus*, Dungeness crab, *Cancer magister*, crayfish, *Orconectes virilis*, and the American lobster, *Homarus americanus*. These findings suggest the possible presence of a carrier process operating in the brush border membrane of digestive organs to transport complete disaccharides into the blood of these invertebrates.

While most vertebrate and mammal research has suggested no sucrose transport, plants are able to transport sucrose. Sucrose is one of the main products of photosynthesis in plants (Bush, 1989, Lemoine et. al 2013, Lunn, 2016). It also serves as an important carbohydrate storage molecule. The product of rapid photosynthesis is glucose, but often plant cell organelles called amyloplasts polymerize glucose into starch using enzymes adenosine diphosphoglucose (ADPG) and ADPG PPase (Zeeman et. al 2007, Geigenberger, 2011). Once synthesized, starch (amylose and amylopectin) is transported from where it is synthesized by photosynthesis in the leaves down to the roots or fruits for storage. Sucrose is initially actively transported into companion cells of

leaf vein phloem cells (Lemoine et., al 2013, Lunn, 2016). From there, diffusion draws sucrose into plant sieve tubes where sucrose flows down its concentration gradient to where it is required in the plant. Next, sucrose is again actively transported into a fruit or root system, creating an osmotic pressure difference that draws water in with it (Bush 1989).

In 2011, a novel animal sucrose transporter (SCRT) gene was identified in *Drosophila melanogaster*, similar to sucrose transporters in plants (Meyer et al. 2011). The authors of this study isolated the SCRT gene from this fly and characterized sucrose uptake kinetics in a yeast expression system, *Saccharomyces cerevisiae*. The complete amino acid sequence of this gene was published recently by the same authors (Vitavska et al. 2013). The role of this protein in nutritional physiology remains to be shown since the function of the protein was not characterized in the original organism in which it was identified. The purpose of the present study was to investigate whether the products (e.g., disaccharides) of incomplete sugar polymer digestion could be transported by an animal membrane. A recent study conducted on the lobster intestine suggested a possible crustacean analog to the *Drosophila* SCRT protein carrier (Likely et al. 2014). Lobster intestine was perfused in a transepithelial flux chamber, and ^{14}C -sucrose was pumped through it. Samples taken from the bath surrounding the intestine contained intact sucrose, as verified by thin layer chromatography. In a second experiment, it was shown that the disaccharide trehalose significantly inhibited sucrose transport across the intestine, suggesting that it utilized the same transporter. Due to these findings, it is possible that incomplete disaccharide digestion and absorption also occurs in the hepatopancreas of this animal.

The lobster hepatopancreas serves similar purposes as the mammal liver, pancreas and intestine. It is a large digestive organ extending from behind the head to the end of the carapace, and is green in color. Food is enzymatically digested and partially absorbed here before moving

into the intestine (Thamotharan and Ahearn, 1996, Duka et. al 2011, Duka et. al 2012). Nutrients are absorbed through cellular brush-border membranes facing the lumen of the hepatopancreas and distributed throughout the body via the blood. It is generally believed that most nutrients are absorbed by the hepatopancreas, leaving minimal concentrations of these substances in the intestine for further transfer to the blood. Preparation of hepatopancreatic brush border membrane vesicles is a useful way to characterize protein transporters present on this membrane involved in the absorption of dietary nutrients.

Complete characterization of a putative SCRT transporter in a crustacean model will greatly expand our knowledge of sugar transport in the animal kingdom. Furthermore, it clarifies whether or not invertebrates must rely on complete hydrolysis of complex carbohydrates to monosaccharides or whether direct nutritional benefit can be attained from sugar polymers such as disaccharides.

MATERIALS AND METHODS

Ethical approval

The present study involves the handling and euthanasia of an invertebrate crustacean species, the American lobster. At present, there are no defined IACUC guidelines for invertebrates. Thus, no protocol review was necessary for this study. Animals were euthanized with cuts to the ganglia between the front walking legs and ventral central nerve cord, and subsequent removal of the heart.

Sampling methods

We utilized the American Lobster, *Homarus americanus*, as the crustacean model organism. For the purpose of standardizing variables, only large, healthy male individuals were utilized throughout the study, and were purchased from Fisherman's Dock, Jacksonville, Florida. Animal history prior to purchase was unknown. The ease of acquiring lobsters and larger body size made these animals highly suitable for this particular study.

Preparation of brush border membrane vesicles (BBMV)

After fasting in a 450 mM sodium-containing saltwater aquarium (10-14°C; pH 8.2) for no more than 24 hours, the entire hepatopancreas was extracted from the lobster by dissection (Figure 1) and purified to brush border membrane vesicles using a salt buffer centrifugation process previously described by Ahearn et al. (1985). Approximately 20 grams of hepatopancreas were put into 60 ml of Buffer 1 containing 300 mM mannitol, 5 mM EGTA, 0.1 mM PMSF, and 12 mM Tris HCL at a pH of 7. The solution was homogenized in a blender for 3 minutes with 240 ml Milli-Q water and centrifuged for 30 minutes at 25,000 rpm (59,000 g) at 4°C. The supernatant

containing cell debris was discarded and the pellets were combined into Buffer 2 containing 300 mM mannitol and 12 mM Tris HCL at a pH of 7. The solution was homogenized and centrifuged for 30 minutes at 25,000 rpm (59,000 g) at 4°C. The supernatant was discarded and the pellets were combined again into Buffer 2. The solution was put on ice for 15 minutes and 600 µl of 10 mM MgCl₂ were added to the solution to remove soluble enzymes released from cellular disruption. After 15 minutes, the solution was centrifuged for 15 minutes at 3,000 rpm (850 g) at 4°C. The supernatant was collected and centrifuged for 30 minutes at 25,000 rpm at 4°C. The pellets were combined into Buffer 3 containing 60 mM mannitol, 5 mM EGTA and 12 mM Tris HCL at a pH of 7. The solution was homogenized, put on ice and 350 µl of 10 mM MgCl₂ were added. After 15 minutes on ice, the solution was centrifuged for 15 minutes at 3,000 rpm at 4°C. The supernatant was centrifuged for 30 minutes at 25,000 rpm at 4°C. The pellet was resuspended in a final transport buffer containing 300 mM mannitol and 12 mM hepes at a pH of 7. The solution was homogenized and centrifuged for 30 minutes at 25,000 rpm at 4°C. The final pellet was suspended in 600 µl transport buffer and a syringe was used to homogenize the pellet. The solution was vortexed and three 10 µl samples were used in a Bradford Standard protein assay at a wavelength of 595 nm to obtain brush border membrane protein concentration in mg/ml protein (Figure 2). These were conducted on each new vesicle preparation before continuing on to transport and kinetic experiments (Figure 3). Three 10 µl samples were added to 5 ml protein dye and 90 µl transport buffer. The blank contained no protein, only 100 µl transport buffer. The samples were read at a wavelength of 595 nm and samples were averaged and units were converted from mg/ml vesicle protein to mg/ 20 µl vesicle protein. A protein standard control curve was produced using bovine serum albumin (BSA) in concentrations ranging from 0 mg/ml to 1.0 mg/ml. Vesicle samples were kept on ice for the remainder of the experiment.



Figure 1. A dissected American lobster, *Homarus americanus*. The hepatopancreas has been removed and is shown to the right.

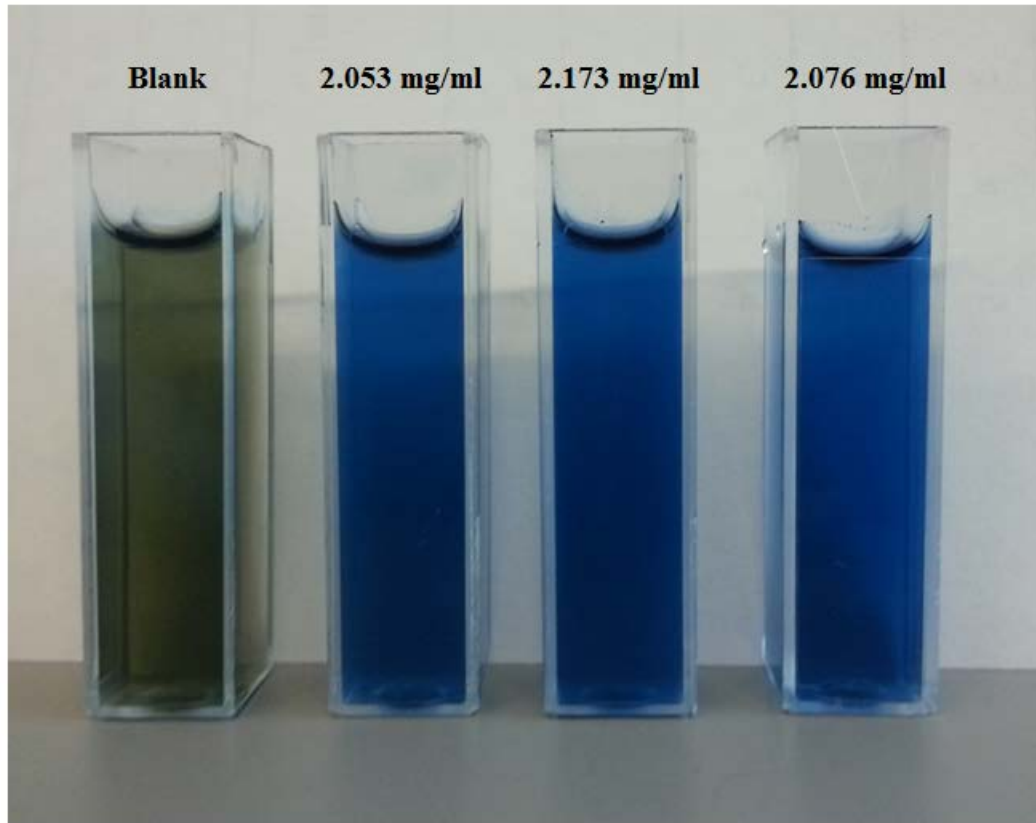


Figure 2. An example of a completed Bradford Standard protein concentration assay.

Identification and characterization of a disaccharide transport carrier

The presence of a functional sucrose transporter (SCRT) was identified using previously described Millipore filtering techniques (Figure 4) (Hopfer et al. 1973). Purified BBMV samples were kept on ice until used experimentally. [^{14}C] Sucrose (435 $\mu\text{Ci}/\text{mmol}$) was used in one-hour time course uptake experiments (3 μl / 140 μl incubation medium). Composition of the incubation medium was subject to change with each set of experiments, but the volumes always remained the same. A 20 μl BBMV sample was added to 120 μl of a 150 mM NaCl, 12 mM HEPES, radiolabeled ^{14}C -sucrose incubation medium at a pH of 7 and mixed by vortexing. After addition of the BBMV, 20 μl samples were extracted at 15 seconds, 1 minute, 2 minute, 5 minute, 10 minute, 30 minute and 60 minute time points from the incubation medium and put immediately into an ice cold choline chloride stop solution and run through Millipore filters (0.65 μm pore diameter) under vacuum to retain vesicles. An additional 3 mL of ice cold stop solution was filtered after every sample to reduce nonspecific isotope that was not accumulated within the vesicles. Washed filter paper was then immediately added to vials containing 3 mL Beckman scintillation cocktail. Samples were counted using a Beckman LS-6500 scintillation spectrometer. A minimum of 3 technical replicates at each time point were used in each experiment.

Each time an experiment was conducted, the protocols for the preparation of brush border membrane vesicles and the isotope vacuum filtering remained the same. Experimental external media that were varied during this project included pH, sodium concentration, potassium concentration, sucrose concentration, and the addition of possible competitive inhibitors such as maltose and trehalose, disaccharide sugars with two glucose moieties and glycylsarcosine, a dipeptide. The latter peptide was used to assess whether ^{14}C -sucrose was transported by a carrier

system that recognized a wide range of substrates (PEPT 1). For additional details concerning each set of experiments, see the results section.

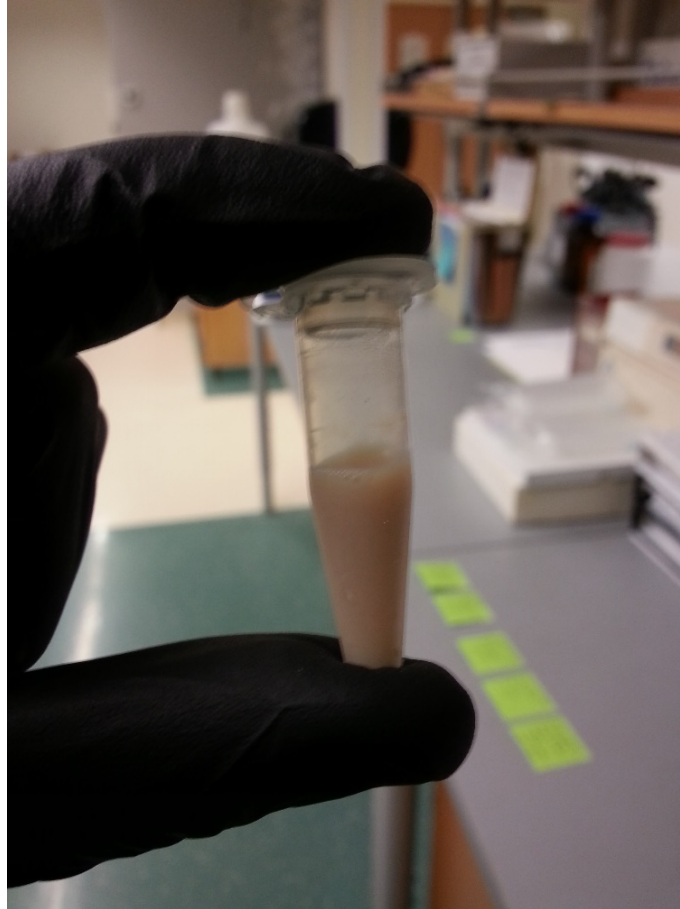


Figure 3. Final purified brush border membrane vesicle protein produced from the hepatopancreas of the American lobster. Approximately 20 grams of hepatopancreas was purified down to 600 μ l BBMV. Purified BBMV was kept on ice throughout transport and kinetic experiments. Samples were taken in 20 μ l volumes.



Figure 4. Basic protocol for a radioactive isotope experiment using brush border membrane vesicles. Radioactive ^{14}C -sucrose (3 μl) was added to the incubation medium (140 μl) containing 20 μl of prepared membrane vesicles. The incubation medium was filtered, then discarded, and vesicles were recaptured using Millipore filter vacuum techniques. Samples were counted using a Beckman LS-6500 scintillation spectrometer.

Statistical analyses

The radioactive CPM-values from each set of replicate scintillation vials were averaged and expressed in units of nanomoles / mg protein \pm 1 SEM. SigmaPlot 10.0 (Systat Software Inc., San Jose, CA) was used to create kinetics graphs from the raw data to be analyzed. Statistical differences in ^{14}C -sucrose uptake were determined using one-way ANOVAs, Repeated Measures ANOVAs for samples taken over a time course, and Tukey's Post Hoc tests.

RESULTS

Possible use of PEPT 1 transporter for ^{14}C -sucrose transport

To establish whether sucrose was being carried by a novel, distinct SCRT disaccharide transporter similar to the SCRT transporter identified in *Drosophila melanogaster*, the possibility of Peptide Transporter 1 (PEPT 1) had to be ruled out as a transport mechanism for sucrose. PEPT 1 is a well-characterized solute carrier for oligopeptides that is found in the kidneys and intestines of a wide range of organisms (Thamotharan and Ahearn, 1996, Verri et al. 2010). It has been shown to carry a wide range of larger biochemical molecules, using a proton-dependent transport mechanism, thus it can be characterized as a cotransporter. A BBMV vesicle experiment was set up using 2.5 mM ^{14}C -sucrose in incubation media containing glycyl-sarcosine, a dipeptide known to be transported by PEPT 1 (Thamotharan and Ahearn, 1996). Concentrations of glycyl-sarcosine ranged from zero to 50 mM. An outside sodium concentration of 150 mM was used. All incubation media and internal vesicle media were kept at a pH of 7. Lack of glycyl-sarcosine inhibition of sucrose uptake into vesicles indicated PEPT1 was not the functional transporter of sucrose (Figure 5). There was no significant difference in the uptake of ^{14}C -sucrose uptake across 0-50 mM glycyl-sarcosine ($P=0.875$). Therefore, higher concentrations of glycyl-sarcosine had no effect on the uptake of ^{14}C -sucrose over a one-minute incubation.

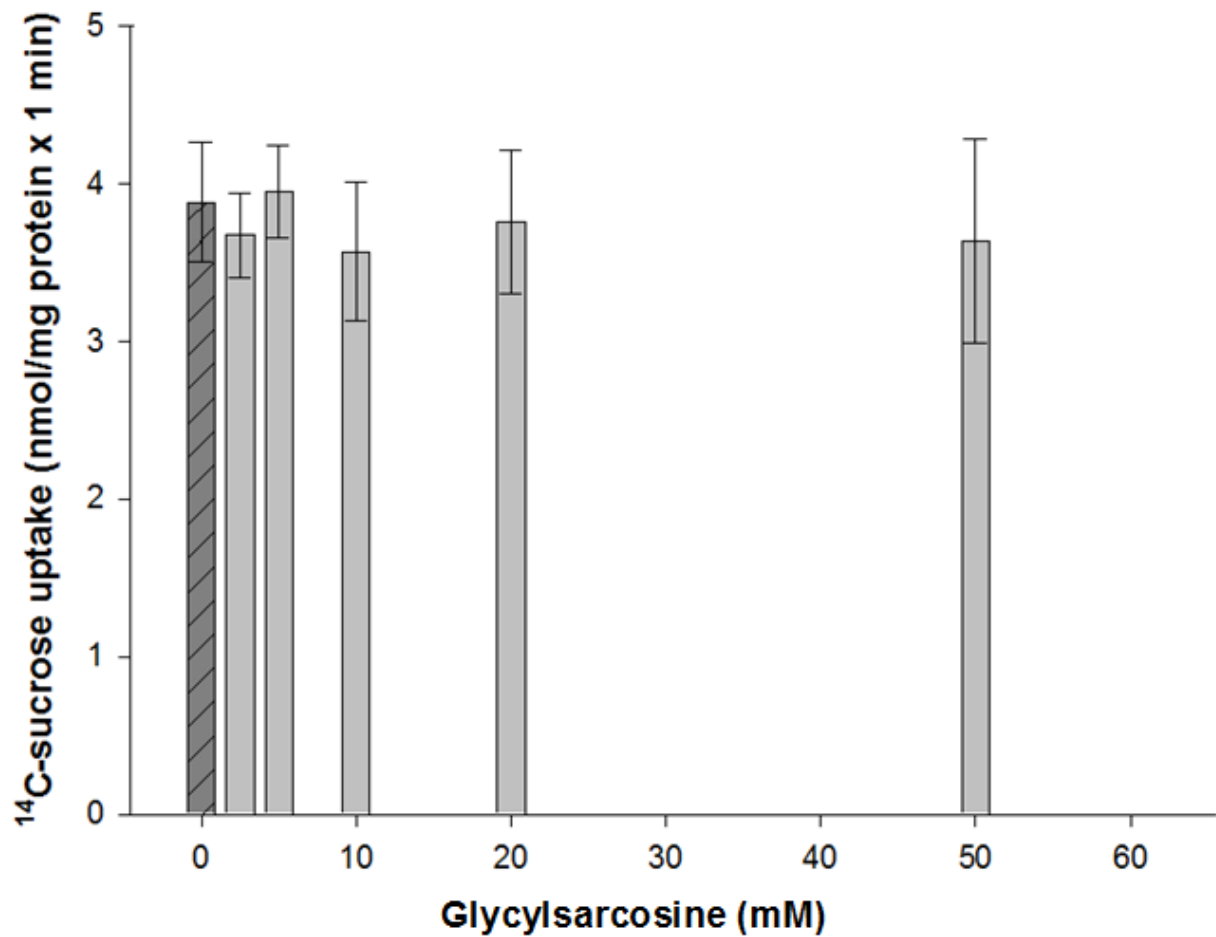


Figure 5. Dipeptide glycyl-sarcosine was used as a possible competitive inhibitor of 2.5 mM ¹⁴C-sucrose over a one-minute incubation period. A constant internal and external BBMV pH of 7 was maintained. There was no significant difference in the uptake of ¹⁴C-sucrose across 0-50 mM glycyl-sarcosine (One Way ANOVA P=0.875). Lack of glycyl-sarcosine inhibition of sucrose uptake indicates PEPT 1 was not the functional transporter of 2.5 mM ¹⁴C-sucrose.

Ion driving forces for ^{14}C -sucrose uptake by hepatopancreatic BBMV

After ruling out PEPT 1 as the transport mechanism for disaccharides in the lobster, a putative distinct transporter analog for sucrose was investigated in the hepatopancreas using a proton gradient as a possible transport driving force for sugar uptake, as was identified in *Drosophila melanogaster* in 2011. The hepatopancreas and stomach of crustaceans reaches a minimum pH of 4 (Gerencser et al. 2004), so a pH of 4 was used as the incubation medium over a one-minute uptake of ^{14}C -sucrose, as well as pH 5, 6 and 7. An acidic pH of 4 showed a significantly higher uptake of sucrose over one minute than pH 5, 6 and 7 (Figure 6). Less extreme proton concentration gradients of 5, 6 and 7 were not significantly different from each other and did not stimulate sucrose uptake into vesicles containing a 300 mM mannitol medium at pH 7.

The possibility of sodium ions acting as a possible driving force in addition to protons for sugar uptake was also investigated using a sodium gradient across BBMV (150 mM sodium), as sodium is the most abundant ion available to lobsters in a marine environment. An experiment was conducted using 150 mM sodium outside and no sodium inside vesicles (Figure 7). A low proton concentration was maintained both inside and outside vesicles at a pH of 7. Uptake of 0.5 mM ^{14}C -sucrose was measured over a time course that went out to two hours. Vesicles were collected at 15 seconds, 1 minute, 5 minutes, 10 minutes, 30 minutes, 60 minutes and 120 minutes. The purpose of utilizing a time course was to look for the presence of an overshoot (^{14}C -sucrose uptake transiently greater inside vesicles than at equilibrium) in a sodium medium, which would indicate that sodium would be considered a driver ion for sugar transport.

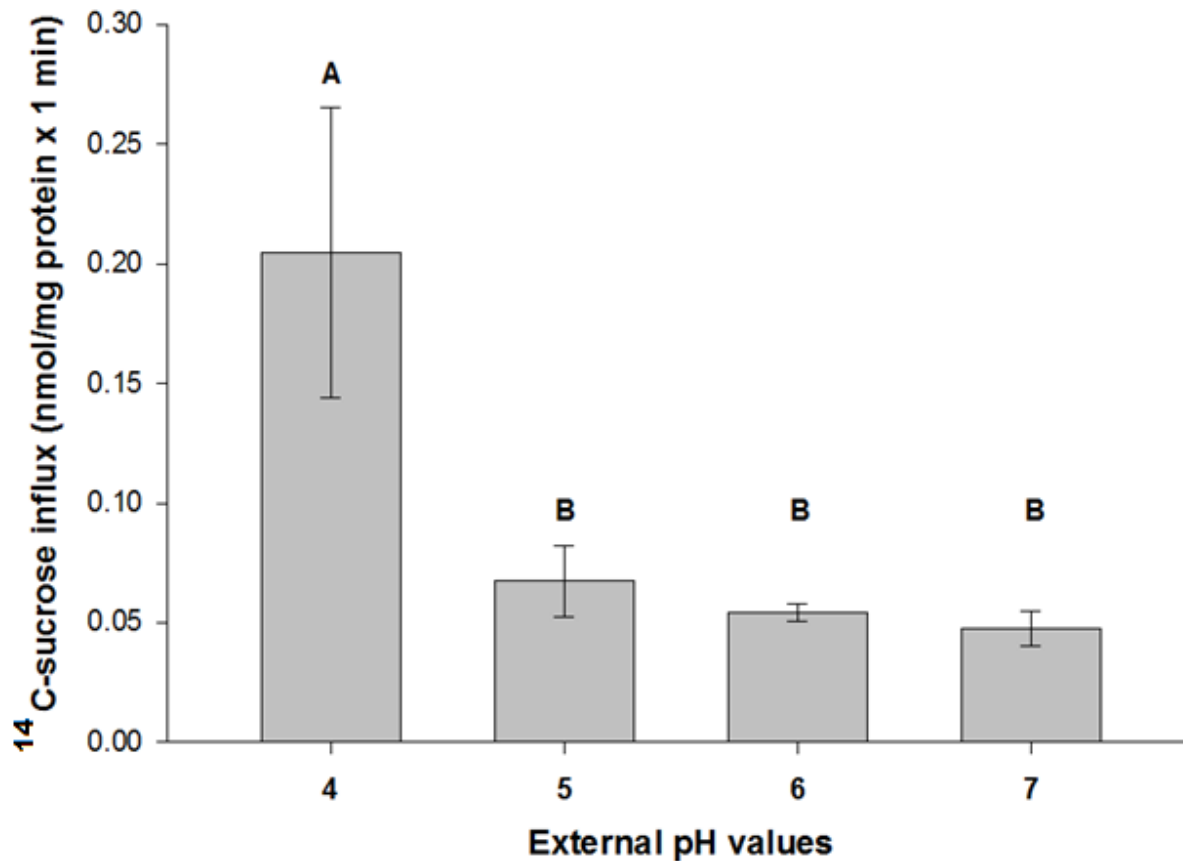


Figure 6. A proton gradient was established across the vesicle membrane using pH 7 inside the vesicles and one of four different external pH values to assess whether a proton gradient could drive 0.05 mM ¹⁴C-sucrose transport into brush border membrane vesicles. No sodium was used in this experiment. The outside and inside media contained 300 mM mannitol and 12 mM hepes. An outside medium at pH 4 showed significantly higher ¹⁴C-sucrose uptake in one minute than incubation media at pH 5, 6 and 7 (one-way ANOVA P=0.006). Lower proton gradients at pH 5, 6 and 7 were not significantly different from each other (one-way ANOVA P=0.938).

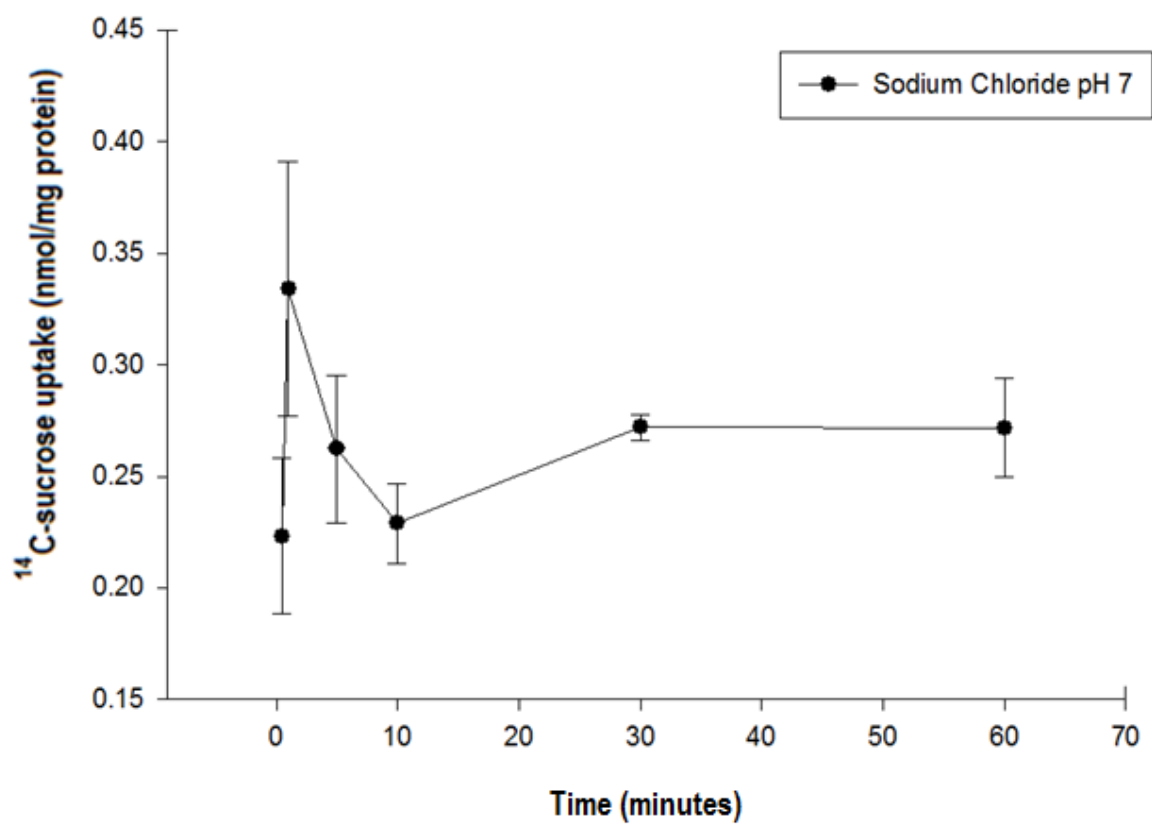


Figure 7. Uptake of 0.5 mM ^{14}C -sucrose using 150 mM sodium chloride in the outside medium.

Internal and external pH across the BBMV membranes remained at a pH of 7.

To establish whether protons and sodium inhibit each other as the main transport mechanism of disaccharides, the uptake of 0.05 mM ^{14}C -sucrose was measured over a one-minute interval in incubation media of decreasing sodium concentration and stable pH of 7. A sodium concentration of 150 mM at a pH of 7 resulted in the highest uptake of ^{14}C -sucrose (Figure 8), and was significantly higher than all other incubation media tested. A second control medium containing 150 mM sodium at pH 4 significantly inhibited ^{14}C -sucrose uptake over one minute. All other incubation media including sodium concentrations 100 mM, 50 mM, 10 mM and 5 mM decreased ^{14}C -sucrose uptake over one minute.

Figure 9 are data taken from Figure 8 and are plotted as the effect of increasing external sodium concentration on the influx of 0.05 mM ^{14}C -sucrose with pH maintained at pH 7. The hyperbolic relationship between the variables indicates that sodium acts as a driver of ^{14}C -sucrose influx by binding to the same carrier as the sugar following Michaelis-Menten binding kinetics and uses its transmembrane concentration gradient across the vesicle membrane to drive in the uptake of the sugar. The kinetic constant values for this relationship are $K_{\text{Na}} = 6.07 \pm 2.35$ mM sodium (sodium concentration resulting in $\frac{1}{2}$ maximal ^{14}C -sucrose influx at 0.05 mM ^{14}C -sucrose) and $J_{\text{max}} = 0.055 \pm 0.005$ nmoles/ mg protein x minute (maximal influx rate of 0.05 mM ^{14}C -sucrose over the chosen sodium concentration range). These results strongly support the use of a membrane carrier protein servicing the transport of ^{14}C -sucrose and sodium.

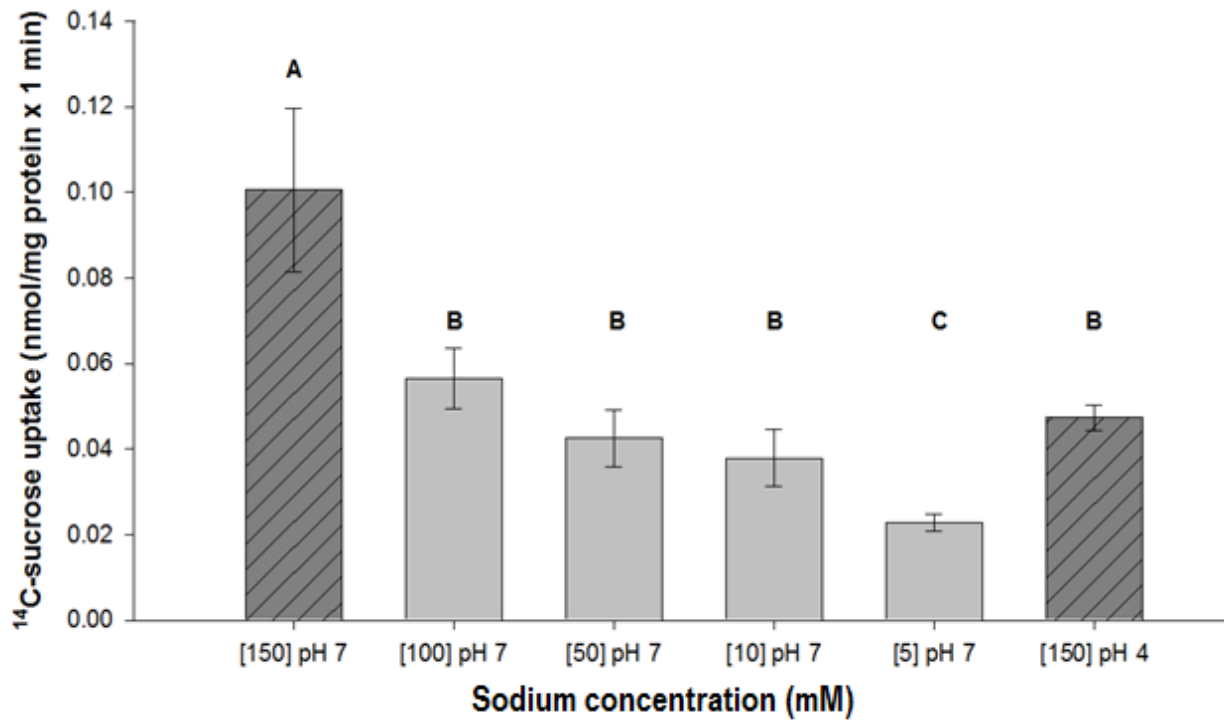


Figure 8. An acidic proton gradient at pH 4 significantly inhibits 0.05 mM ¹⁴C-sucrose uptake over one minute by 150 mM sodium (P=0.004). A control incubation medium containing 150 mM sodium at a neutral pH of 7 showed significantly higher uptake over one minute than all other media tested (P=0.006). Incubation media with decreasing sodium concentration (100-5 mM) showed decreasing ¹⁴C-sucrose uptake.

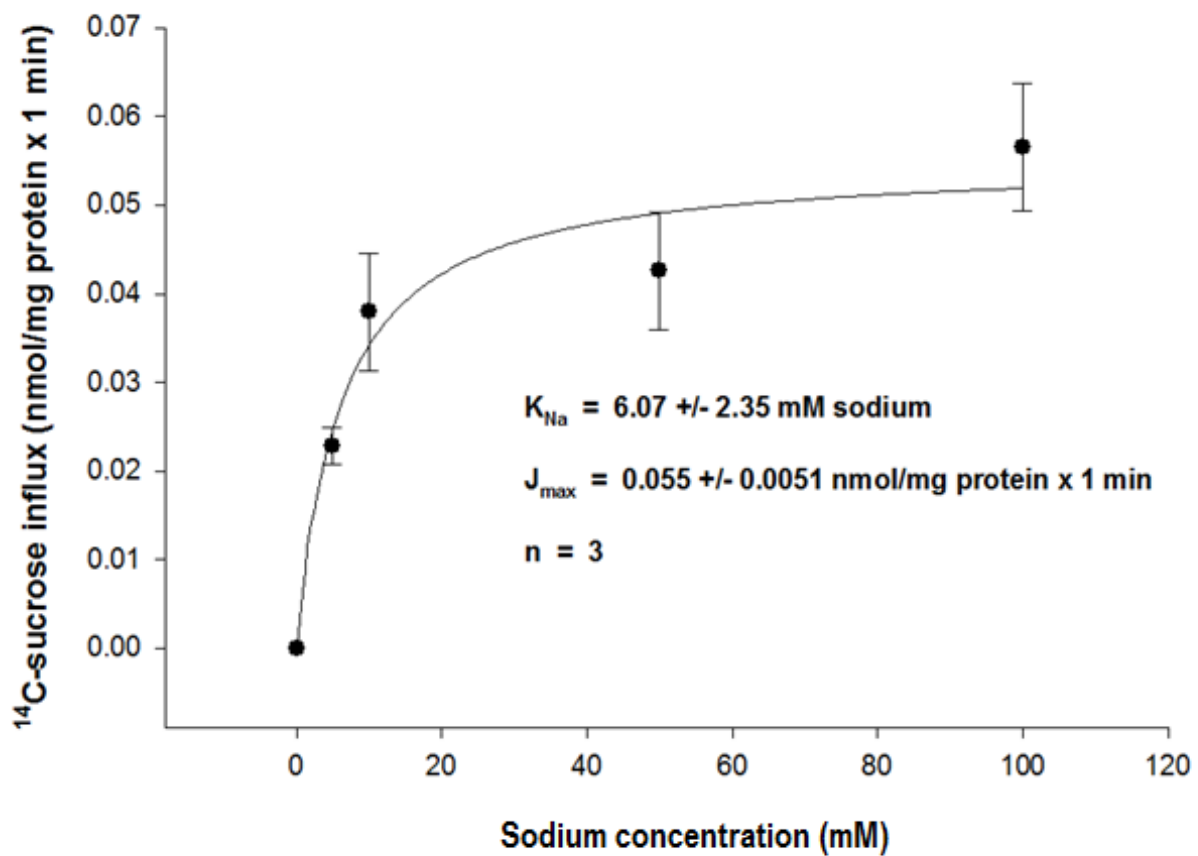


Figure 9. Sodium was investigated as a possible driver mechanism for sugar transport and was shown to stimulate uptake of 0.05 mM ^{14}C -sucrose. Concentrations of sodium ranging from 0-100 mM increased sucrose uptake accordingly. A low proton gradient was maintained with an internal and external vesicle pH of 7. Data were obtained from Figure 8.

Effects of monosaccharides, disaccharides and trisaccharides on BBMV ^{14}C -sucrose uptake

Once sodium ions and protons were established as sugar driving mechanisms into BBMV, and it was shown that sucrose was making it across the membrane intact (Likely et al. 2014), the likelihood of competitive inhibition by other complex and simple carbohydrates was investigated using a wide variety of monosaccharides, disaccharides and trisaccharides on the one minute uptake of 0.05 mM ^{14}C -sucrose. Three monosaccharides including glucose, fructose and galactose were used (0.05 mM). Four disaccharides including trehalose, maltose, lactose and cellobiose were used (0.05 mM). Two trisaccharides including melezitose and raffinose were used (0.05 mM). Results using a variety of monosaccharides and polysaccharides indicated that the disaccharides maltose and trehalose were the only sugars to significantly inhibit sucrose transport (one way ANOVAs, maltose $P = 0.017$, trehalose $P = 0.023$), suggesting a high degree of specificity of the transporter (Figure 10). A previous study conducted by Likely et al. (2014) also showed trehalose to be an effective inhibitor of ^{14}C -sucrose.

Once it was shown that disaccharides trehalose and maltose significantly inhibited 0.05 mM ^{14}C -sucrose uptake into BBMV over one minute with a 150 mM sodium gradient, the type of inhibition by maltose on ^{14}C -sucrose transport was more thoroughly investigated in a second experiment using 20 mM maltose and ^{14}C -sucrose concentrations ranging from 0-0.5 mM (Figure 11). In this experiment all one minute ^{14}C -sucrose influx values in the presence of maltose were lower than their respective control condition lacking maltose. Both the control influx curve lacking maltose and the curve including maltose were hyperbolic functions of their respective sucrose concentrations and followed Michaelis-Menten carrier-mediated transport kinetics. Sucrose influx in the absence of maltose displayed a K_m of 0.1951 ± 0.0630 mM sucrose and a J_{\max} of 0.5884 ± 0.0823 nmol/mg protein x 1 minute (Table 1). Sucrose influx in the presence of 20 mM maltose

had a K_m of 0.5847 ± 0.1782 mM sucrose ($P = 0.030$) and a J_{max} of 0.6536 ± 0.1238 nmol/mg protein x 1 minute ($P = 0.006$) (Table 1). ANOVA P-values indicate the difference between the sucrose control curve and the maltose curve. The highly significant difference between the K_m values of the control sucrose curve and the maltose curve suggests competitive inhibition between the two sugars (Table 1).

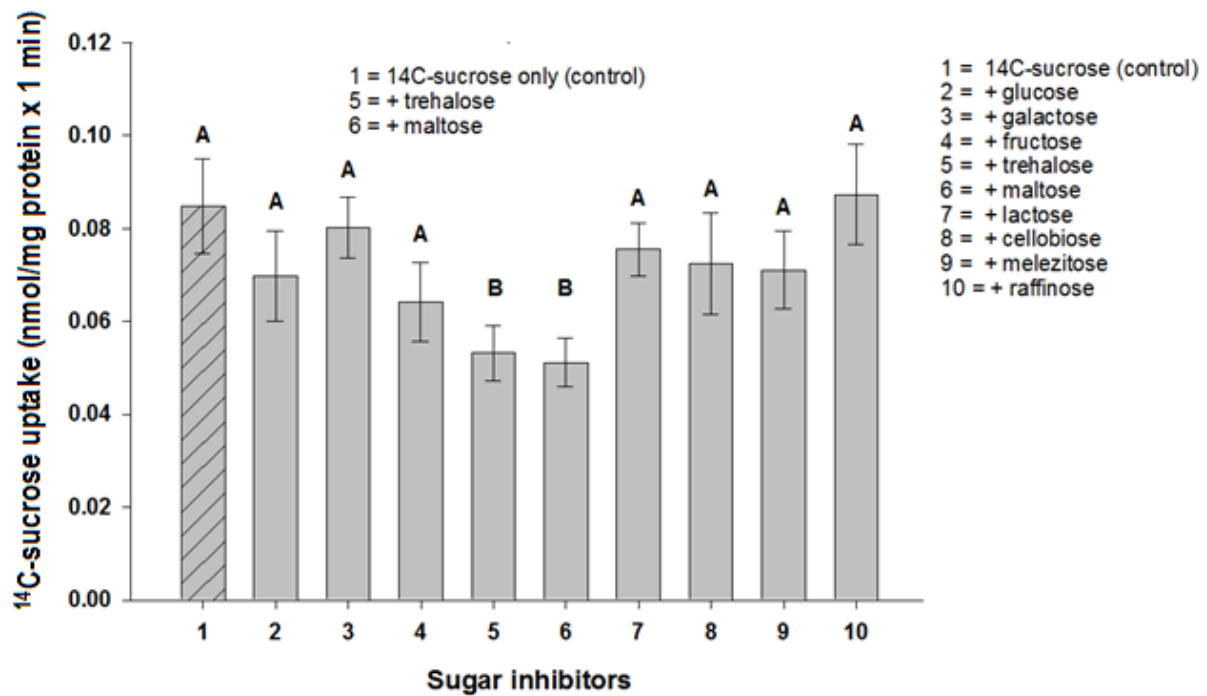


Figure 10. Results using a variety of monosaccharides and polysaccharides (0.05 mM) as possible competitive inhibitors of one minute- 0.05 mM ^{14}C -sucrose transport indicated that the disaccharides maltose and trehalose were the only sugars to significantly inhibit sucrose transport (one way ANOVAs, maltose $P = 0.017$, trehalose $P = 0.023$).

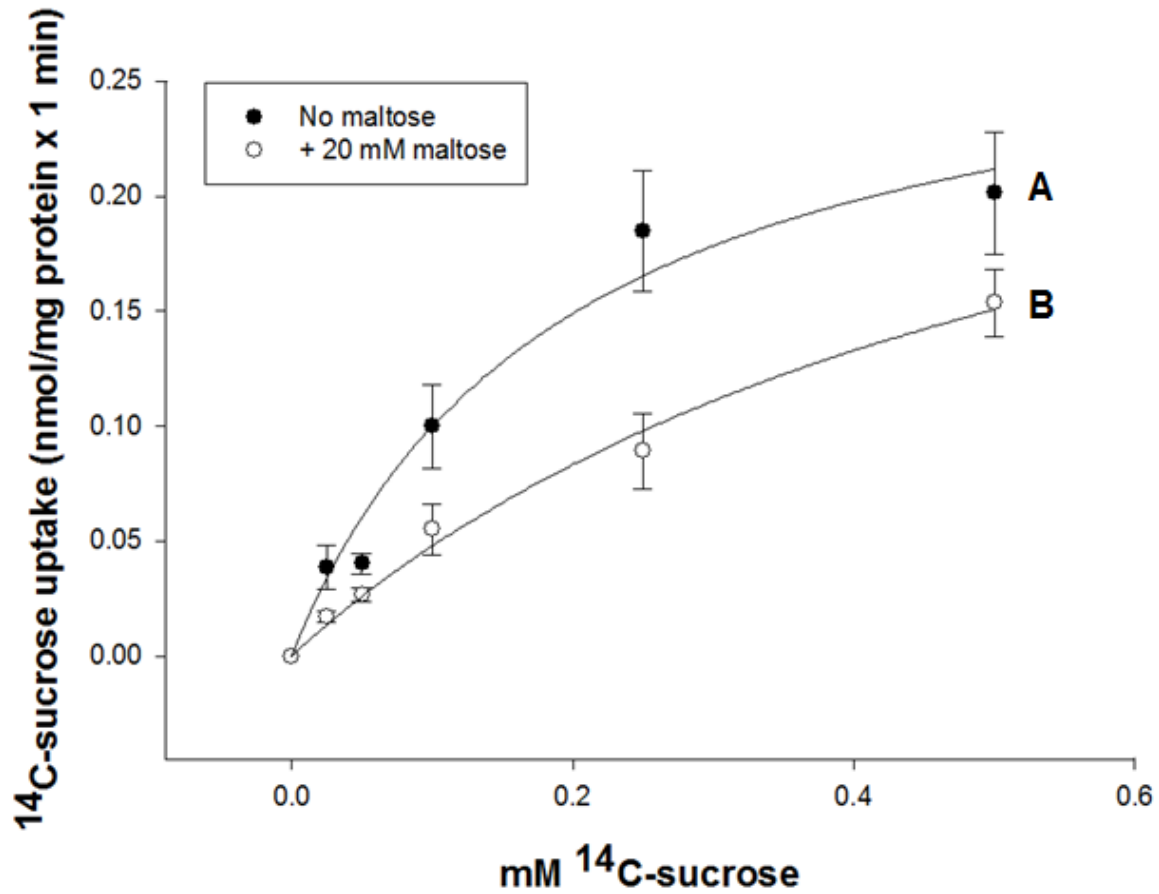


Figure 11. Further investigation into maltose as a possible inhibitor of 0-0.5 mM ^{14}C -sucrose in a medium containing 150 mM sodium at a pH of 7 across the vesicle membrane shows that 20 mM maltose significantly inhibits sucrose transport into vesicles (one-way ANOVA, $P = 0.0304$).

Table 1. Kinetic effect of 20 mM maltose on the influx of ^{14}C -sucrose (0-0.5 mM) in the presence of a 150 mM sodium gradient directed into the vesicles with media at pH 7 on both sides of the membrane (Data from Figure 11). ANOVA P values indicate the difference between the sucrose control curve and the maltose curve.

^{14}C -sucrose uptake (nmol/mg protein) over 1 minute	J_{max}	K_{m}
0.05 mM sucrose control	0.5884±0.0823	0.1951±0.0630
0.05 mM sucrose + 20 mM maltose	0.6536±0.1238	0.5847±0.1782
One way ANOVA P-value between the two treatments	P= 0.774	P= 0.0304

DISCUSSION

The discovery of a gene from *Drosophila melanogaster* that appears to code for a disaccharide transporter and is involved in the nutritional uptake of carbohydrates in the hindgut of this animal is significant, and led to this and other recent studies (Meyer et al., 2011). The goal of this study was to investigate whether the products (e.g., disaccharides) of incomplete sugar polymer digestion could be transported by an animal membrane. A previous study from Likely et al. (2014) provides evidence of a disaccharide SCRT transporter in the intestine of the American lobster, and shows through thin layer chromatography techniques that sucrose, a large disaccharide, is found completely intact in the bath containing a perfused intestine with ^{14}C -sucrose (Figure 12). This indicates the presence of a protein carrier that can facilitate the transport of disaccharides including not only sucrose, but also trehalose, which exhibits competitive inhibition of sucrose over a time course experiment set up (Figure 13).

Carbohydrates, especially the monosaccharide glucose, have been extensively studied and are critical to energy production in a wide array of organisms. Monosaccharides glucose, fructose and galactose are readily available for transport into the blood via monosaccharide transporters imbedded in the brush border membrane of the intestine and other digestive organs that aid in absorption of nutrients to the bloodstream. These transporters include sodium-glucose linked transporters (SGLTs) and sodium-independent glucose transporters (GLUTs) (Meyer et al. 2011, Vitavska et al. 2013) and, in humans, belong to two larger carbohydrate transport families, the human sodium glucose cotransporter family (SLC5) and the major facilitator superfamily of membrane transporters (SLC2) respectively. After food is broken down via enzymatic hydrolysis along the luminal wall of the intestine, the smallest subunits can be transported through the brush border and basolateral cell walls into the blood of the animal.

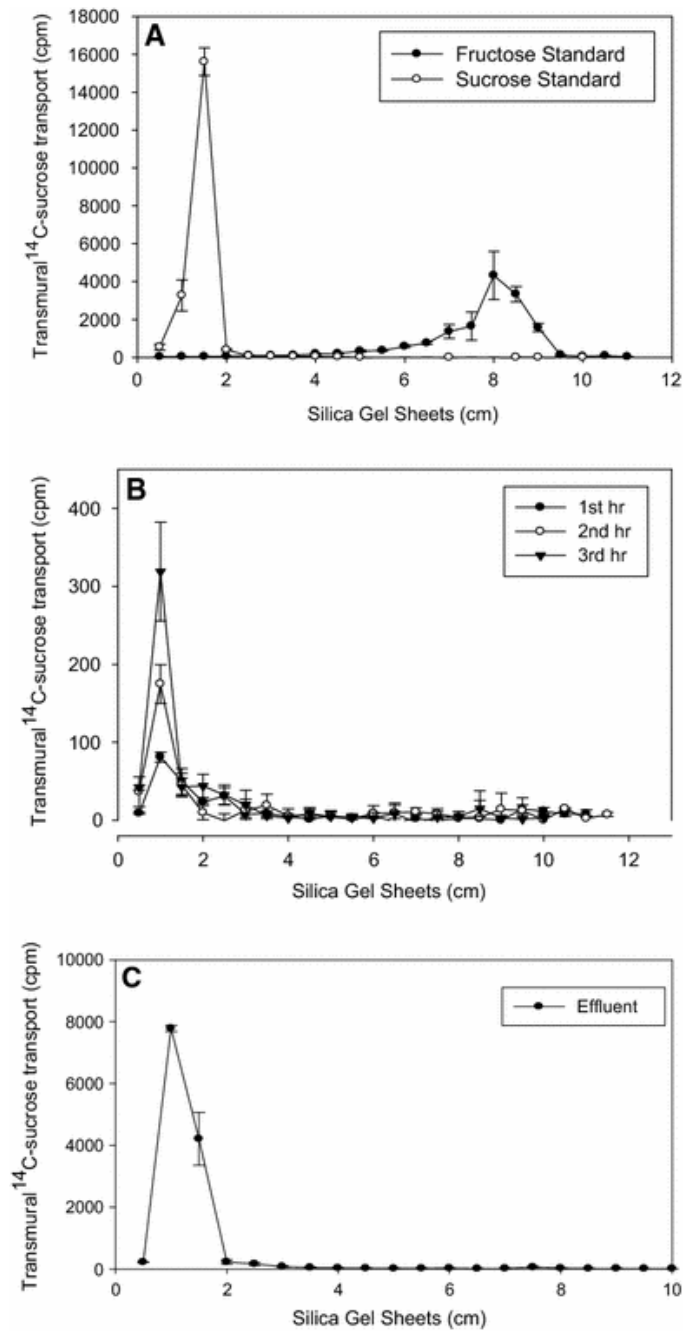


Figure 12. Thin-layer chromatography of ^{14}C -sucrose and ^3H -D-fructose standards (a), samples of the serosal bath after 1, 2, and 3 h perfusions with ^{14}C -sucrose (b), and samples of the perfusate effluent after 3 h perfusions (c). Each experiment using 5 mM ^{14}C -sucrose was conducted a total of four times on four separate animals. (From Likely et al., 2014)

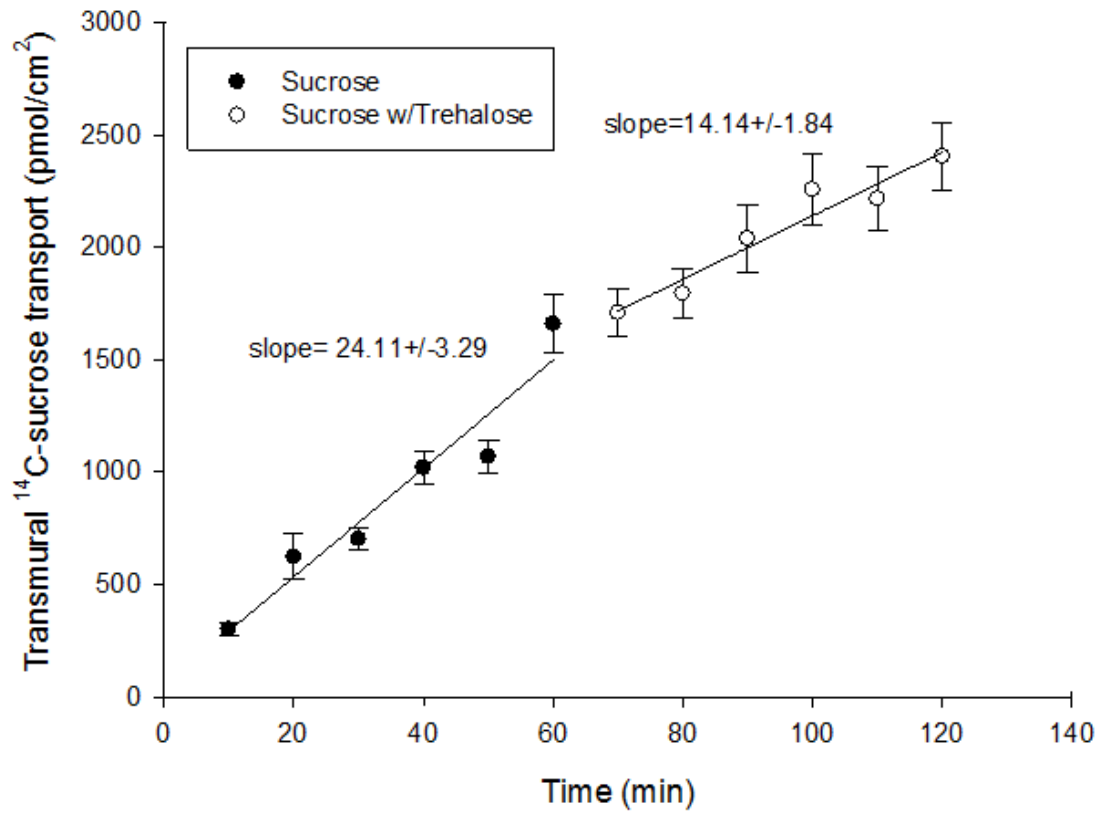


Figure 13. Effect of 5 mM luminal trehalose on transmemural 0.1 mM ^{14}C -sucrose transport across lobster intestine. After one hour, addition of 5 mM trehalose significantly competitively inhibits uptake of ^{14}C -sucrose ($P < 0.05$). (From Likely et al., 2014)

Up until 2011, transport of intact disaccharides across animal membranes had not been reported. The discovery of a gene in *Drosophila melanogaster* that coded for a novel sucrose transporter (SCRT) was the first documented disaccharide transporter outside of the plants, which are able to actively transport photosynthetic products including sucrose from the leaves of the plant down to the root system as one of the main carbohydrate storage mechanisms (Lemoine et., al 2013).

The mechanism by which SCRT in plants aids sucrose transport from one source location in the plant to another potentially distant sink location is shown in Figure 14. Leaf mesophyll cells synthesize sucrose during photosynthesis and transfer it into companion cells (Swan, 2012). From companion cells the sugar is actively transported into phloem cells. Phloem tubes are next to xylem tubes, which supply the plant with water. Due to a now higher sugar concentration in the phloem, water from the xylem flows via osmosis from the xylem to the phloem down its concentration gradient (Swan, 2012, Lemoine et., al 2013). As a result, fluid carrying sugars are driven away from the sugar source down a plant using a pressure flow gradient.

Graphic redacted, paper copy
available upon request to home
institution.

Figure 14: Transport of synthesized sucrose in plants from leaf cells through companion cells to phloem for distribution throughout plant ending in storage site in root (Swan, 2012).

Graphic redacted, paper copy available upon request to home institution.

Figure 15: Role of secondary active transport of sucrose with protons to facilitate the accumulation of sucrose from leaf mesophyll cells to phloem cells for transfer throughout the plant. A primary active transport proton pump transfers hydrogen ions up a concentration gradient from companion cells to sieve tube elements and the accumulated protons are used to drive sucrose up its concentration gradient from the companion cells into the phloem cells using the proton gradient as a driving force. Accumulated sucrose in phloem is distributed to sugar storage sites in plants (Swan, 2012).

The mechanism by which sucrose is actively transported from companion cells into phloem cells is displayed in Figure 15, which shows the presence of the SCRT transporter. In Figures 14 and 15, companion cells share membranes with mesophyll and phloem cells. Secondary active sucrose transport occurs across both membranes using the combination of a primary active proton pump and a SCRT proton-sucrose cotransporter. This combination results in an accumulation of protons in companion cells and an accumulation of sucrose in phloem cells. Sucrose is then transported throughout phloem tissues using water transferred into phloem from xylem tissues.

Likely et al. (2014) proposed that an analog to the SCRT found in *Drosophila melanogaster* also existed in the intestine of the American lobster. This model has now been

expanded to include the brush border membrane of the hepatopancreas of the American lobster (Figure 16). The SCRT identified in the intestine of the lobster has also been characterized in the brush border membrane of the hepatopancreas and works by a similar mechanism. Whereas the previous model only showed trehalose as competitive inhibitor of sucrose, we now have evidence that another disaccharide, maltose, also acts to inhibit sucrose, indicating that it too uses this SCRT. Figure 10 shows that 0.05 mM ^{14}C -sucrose was inhibited by approximately 50% by 20 mM trehalose and maltose. A higher concentration of the inhibitory sugars would likely block further transport of ^{14}C -sucrose. The fact that ^{14}C -sucrose transport K_m was found to be 0.2 mM, while 20 mM inhibitory sugars only inhibited ^{14}C -sucrose transport by 50% suggests that the relative affinity of the proposed SCRT in lobster is significantly higher for sucrose than for the other two disaccharides. This might indicate that the two potential dietary sugars, trehalose and maltose, naturally occur at relatively high concentrations in the food eaten by lobsters. The model of sugar transport across the brush border membrane of the hepatopancreas has been expanded to include this new evidence.

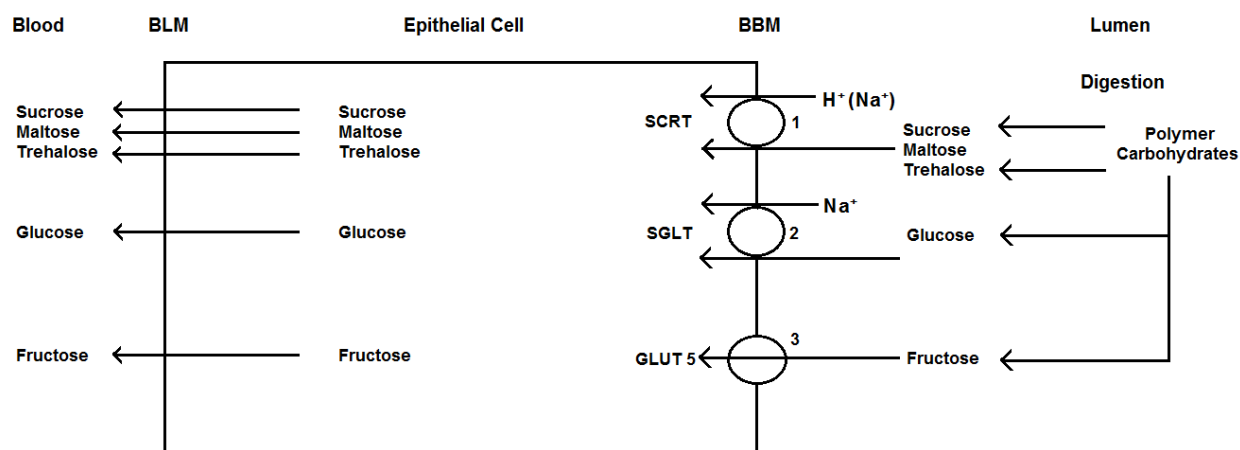


Figure 16. A proposed updated model of carbohydrate transport in the brush border membrane of the American lobster hepatopancreas. The above model for sugar transport in hepatopancreatic epithelial brush border membrane includes the presence of at least three separate sugar carrier proteins. Previous studies have shown the presence of a sodium dependent SGLT1-like transporter for glucose uptake and a GLUT5 transporter for fructose influx. New research from this thesis now adds a third sugar transporter to the brush border that transfers disaccharides from lumen to cell cytoplasm and is tentatively identified as a lobster SCRT. This disaccharide transporter is shared by sucrose, trehalose, and maltose and uses either a proton or sodium gradient as driving forces for secondary active transport.

Results from this study indicate that well-characterized peptide transporter 1 (PEPT 1) is not the transport mechanism utilized by the disaccharide sucrose to get into the hepatopancreatic epithelial cells. Lack of glycyl-sarcosine inhibition of 2.5 mM ^{14}C -sucrose uptake (Figure 5) supports this hypothesis. Because these high concentrations of glycyl-sarcosine do not significantly inhibit sucrose uptake into BBMV, there must be another transport mechanism transferring sucrose to the blood.

While investigating other possible transport mechanisms, the present study has shown that both a proton gradient and a sodium gradient across the hepatopancreatic vesicle membranes are capable of driving sucrose transport. Protons (pH 4) can drive sucrose across BBMV in the absence of other drivers like sodium. A luminal pH of 4 has been shown to be the most effective, resulting in significantly higher uptake values of ^{14}C -sucrose transport compared to lower proton gradients seen at pH 5, 6 and 7 (Figure 6). This may be due to the fact that in crustaceans, the stomach and the hepatopancreas reach a pH of 4, which may maximize transport of nutrients into the bloodstream. Differences in sucrose uptake were consistently seen between pH 4 and higher pHs of 5, 6 and 7, but in all cases, sucrose uptake in media a pH 5, 6 and 7 were not significantly different from each other, suggesting they were all not very effective at driving sucrose transport across the membrane.

While investigating the possibility of the sodium ion as a possible driver mechanism for sucrose, results showed that if sodium was added at 10 times the concentration of sucrose, sodium can be utilized as an effective driver of sucrose into BBMV (Figures 7-9). However, protons can inhibit the uptake of sucrose in media containing sodium. Both protons and sodium may be used as transport drivers under different conditions. Sodium is highly abundant in the marine environment in which American lobsters and other marine crustaceans live, so it makes sense that

it would be utilized as an energy efficient way to transport large sugars like sucrose, trehalose and maltose into BBMV. However, as previously discussed, the acidic conditions inside the stomach and hepatopancreas may explain how protons can also be utilized to transport disaccharides into the BBMV and from there to the blood of the animal. Therefore, at a non-optimum pH, sodium may be used as a driver mechanism of disaccharides.

A potentially biologically relevant reason why both sodium and protons are able to independently drive the uptake of ^{14}C -sucrose into hepatopancreatic BBMV may relate to the feeding style of crustaceans in general. Crustaceans are “messy eaters” tending to rip apart food prior to its ingestion with significant amounts of seawater entering the animal with food material. Initial entry of food into the stomach-hepatopancreas luminal digestion corridor would likely have a high sodium concentration and a pH near that of seawater (e.g., pH 8.1). Under these conditions, initial transport of dietary sucrose into hepatopancreatic epithelial cells would use sodium as a driver co-substrate because sodium is at a high concentration and protons are at a low concentration. As digestion proceeds, more protons are secreted into the digestion corridor and likely replace sodium as a more effective cotransport substrate. This concept suggests that carbohydrate digestion and absorption follow a sequential process where alternative driver ions power sugar uptake as their luminal concentrations change over time.

Just like the proposed sequential replacement of one cation as a sucrose transport driver by another in the digestive tract, the well-known D-glucose cotransporter in mammals and other animals, including lobsters, shows a sequential replacement of driver cations. In 1997, Hirayama et al. (1997) showed that sodium and hydrogen ions were capable of being co-transported by the SGLT1 carrier protein in mammals. The mammalian intestine had different binding affinities for the cations and appeared to use sodium and protons at different parts of the small intestine during

absorption. It was suggested by these authors that chyme leaving the stomach and entering the duodenum, the first place where the SGLT1 occurs along the gut and functions to transport the sugar from gut lumen to blood, likely uses protons as a driver ion. This is because the pH of the chyme leaving the stomach is considerably acidic until it is neutralized by sodium bicarbonate arriving from secretions of the pancreas. As the chyme moves along the small intestine at a neutral pH, the relatively high sodium concentration in the lumen replaces protons as the predominant sugar absorption driver and this process continues down the remaining length of the organ. Therefore, the replacement of one driver cation by another is not unique to the model presented in this thesis, but it is the first time this concept has been applied to sucrose absorption by a crustacean digestive tract.

The relative binding affinity (K_m) kinetic constant of sucrose transport in the hepatopancreas of the American lobster ($195 \pm 63 \mu\text{M}$) fits well within the table of known K_M values in absorptive organs across species (Table 2). Likely et. al (2014) found that the intestine of the lobster had a much lower K_m ($20.5 \pm 6 \mu\text{M}$), thus a higher affinity for sucrose in the intestine, which is posterior to the main absorptive organ, the hepatopancreas. The affinity of the SCRT for sucrose in the hepatopancreas may be lower due to higher concentrations of sucrose and other disaccharides maltose and trehalose found in this organ. Data in Table 2 from a variety of species of animals suggests that anterior digestive tract organs contain relatively higher concentrations of dietary nutrients than found in posterior portions of the guts from the same animals and display lower K_m values than occur in the posterior parts of the digestive tracts. This arrangement would seem appropriate for maximizing nutrient uptake as food elements pass from one end of the gut to the other. Anterior locations, with their low affinity transporters might remove up to 90% of nutrients consumed, while the remaining 10% would be absorbed by transporters with higher

binding affinities. This arrangement would conceivably leave little dietary nutrient to escape in the feces. Such a sequential absorption process leading to maximal overall substrate absorption is seen in the human renal proximal tubule where two SGLT sodium-glucose cotransporters occur side by side with the lower affinity carrier found immediately after the filtering glomerulus, removing the bulk of filtered glucose, and the higher affinity carrier more distal to the glomerulus and absorbing the remaining filtered sugar before its loss in the urine (Turner and Moran, 1982). This sequential arrangement of similar SGLT carriers with only a difference in relative binding affinity for glucose is able to remove 99.9% of filtered glucose. Sequential nutrient absorption may be a general physiological phenomenon and not restricted only to sugars.

Table 2. Kinetic constants of sugars in absorptive organs across species. The present study adds to this table the K_M value for sucrose in the hepatopancreas of the American lobster (original table found in Likely et. al, 2014).

<u>Species</u>	<u>Tissue</u>	<u>Substrate</u>	<u>K_M</u>	<u>Reference</u>
<u>Invertebrates</u>				
<i>Homarus americanus</i> (lobster)	Hepatopancreas	D-glucose	620±50 μM	Wright et. al, 1997
		D-fructose	325±81 μM	Sterling et. al, 2009
		Sucrose	195±63 μM	Present Study
	Intestine	D-glucose	15.2±3.5 μM	Obi et. al, 2011
		D-fructose	10.1±1.9 μM	Obi et. al, 2011
		Sucrose	20.5±6.0 μM	Likely et. al, 2014
<i>Callinectes sapidus</i> (Blue crab)	Intestine	D-glucose	87±19 μM	Chu, 1986
<i>Macrobrachium rosenbergii</i> (Freshwater prawn)	Intestine	D-glucose	170 μM	Ahearn and Maginniss, 1977
<u>Fishes</u>				
<i>Sebastes caurinus</i> (Copper rock fish)	Pyloric caeca	D-glucose	580±120 μM	Ahearn et. al, 1992
	Intestine	D-glucose	140±20 μM	Ahearn et. al, 1992
<i>Oreochromis mossambicus</i> (Tilapia)	Upper intestine	D-glucose	670±150 μM	Reshkin and Ahearn, 1987
	Lower intestine	D-glucose	390±50 μM	
<u>Plants</u>				
<i>Beta vulgaris</i> (sugar beet)	Root	Sucrose	2,200±150 μM	Willenbrink and Doll, 1979
<i>Saccharum officinarum</i> (sugar cane)	Phloem	Sucrose	826±203 μM	Reinders, et. al, 2012; Reinders et. al, 2006

Experiments using a variety of monosaccharides and polysaccharides indicated that the disaccharides maltose and trehalose were the only sugars to significantly inhibit sucrose transport (Control $K_m = 0.1951 \pm 0.0630$ mM sucrose, $J_{max} = 0.5884 \pm 0.0823$ nmol/mg protein x 1 minute), suggesting specificity of the transporter (maltose $P = 0.017$, trehalose $P = 0.023$). Sucrose in the presence of 20 mM maltose had a K_m of 0.5847 ± 0.1782 mM sucrose ($P = 0.030$) and a J_{max} of 0.6536 ± 0.1238 nmol/mg protein x 1 minute ($P = 0.006$). ANOVA P-values indicate the difference between the sucrose control curve and the maltose curve. The highly significant difference between the K_m values of the control sucrose curve and the maltose curve suggests competitive inhibition between the two sugars. Together, these two disaccharides, utilizing the same transporter, are appropriate for the physiology of the animal in this case, as lobsters commonly digest glycogen and chitin, polymers of maltose and trehalose, respectively. These findings suggest there is a hepatopancreatic carrier process that may function to absorb disaccharides that occur from digestion of naturally-occurring dietary constituents.

The finding that disaccharides occurred in the blood of crustaceans using paper chromatography (Telford, 1968), coupled with the present results of an uptake mechanism removing disaccharides from the gut lumen, and transepithelial transport of intact disaccharides across the intestine (Likely et al., 2014), suggests that circulating complex sugars in the blood of these animals may have a role other than that used by monosaccharides in the same animals. Whereas simple sugars provide immediate energy to body cells, disaccharides involving biochemical pathways to glycogen and chitin synthesis may be retained in a more complex form that can be delivered rapidly to sites of polymer production such as incorporation into new exoskeleton synthesis or glycogen storage forms of glucose.

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